

IL-9 Regulates Allergen-Specific Th1 Responses in Allergic Contact Dermatitis

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The cytokine IL-9, derived primarily from T-helper 9 (Th9) lymphocytes, promotes expansion of the Th2 subset and is implicated in the mechanisms of allergic asthma. We hypothesize that IL-9 also has a role in human allergic contact dermatitis (ACD). To investigate this hypothesis, skin biopsy specimens of positive patch-test sites from non-atopic patients were assayed using quantitative PCR and immunohistochemistry. The cytokines IFN- γ , IL-4, IL-17A, IL-9, and PU.1, a Th9 associated transcription factor, were elevated when compared with paired normal skin. Immunohistochemistry on ACD skin biopsies identified PU.1⁺CD3⁺ and PU.1⁺CD4⁺ cells, consistent with Th9 lymphocytes, in the inflammatory infiltrate. Peripheral blood mononuclear cells from nickel-allergic patients, but not nonallergic controls, show significant IL-9 production in response to nickel. Blocking studies with mAbs to HLA-DR (but not HLA-A, -B, -C) or chloroquine significantly reduced this nickel-specific IL-9 production. In addition, blockade of IL-9 or IL-4 enhanced allergen-specific IFN- γ production. A contact hypersensitivity model using IL-9^{-/-} mice shows enhanced Th1 lymphocyte immune responses, when compared with wild-type mice, consistent with our human *in vitro* data. This study demonstrates that IL-9, through its direct effects on Th1 and ability to promote IL-4 secretion, has a regulatory role for Th1 lymphocytes in ACD.

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INTRODUCTION

After activation, naive CD4⁺ T-helper (Th) cells differentiate along separate lineages into effector T-cell subsets depending on the type of antigen recognized and the selection of cytokines present within the adjacent extracellular milieu. The traditional Th1 or Th2 paradigm has expanded to include Th17, Th9, Treg, and Th22 cells, each with lineage-specific transcription factors and production of a distinct array of cytokines (Wan and Flavell, 2009). Our understanding of the interplay between these cells and their involvement in immune pathology is still incomplete. IL-9 is classically considered to

be associated with the Th2 subset. However, recently an IL-9-producing T-cell subset, named Th9, has been recognized and is implicated in the pathogenesis of allergic inflammation (Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008).

IL-9 is shown to have pleiotropic cellular effects involved in promoting allergic inflammation, including inducing mast cell growth and survival, eotaxin production, and goblet cell metaplasia (Vermeer *et al.*, 2003; Gounni *et al.*, 2004; Kearley *et al.*, 2011). A significantly elevated level of IL-9 has been found in bronchial biopsies from patients with atopic asthma compared with matched controls, and thus IL-9 may have a significant role in the chronic airway inflammation and bronchial hyperresponsiveness seen in asthmatic disease (Ying *et al.*, 2002). Blocking IL-9 inhibits airway remodeling and mastocytosis in a model of chronic lung inflammation (Kearley *et al.*, 2011). A recent study shows that Th9 cells are generated *in vivo* after epicutaneous sensitization by patch application with ovalbumin in mice (Lin *et al.*, 2012). PU.1, an ETS (E26 transformation specific or E-twenty-six)-family transcription factor, has since been identified as a main regulator of IL-9 production in human Th9 cells. Increased PU.1 expression is associated with a decrease in Th2 cytokines and increased phenotypic characteristics consistent with Th9 cells, including production of IL-9. In addition, less allergic inflammation was seen in the lungs of mice with PU.1-deficient T cells compared with their wild-type (WT) counterparts (Chang *et al.*, 2010). Despite their known role in allergic lung disease, their participation in allergic contact dermatitis (ACD) has not been established.

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Abbreviations: ACD, allergic contact dermatitis; APC, antigen-presenting cell; CHS, contact hypersensitivity; cDNA, complementary DNA; DNFB, dinitrofluorobenzene; DNP, dinitrophenyl; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IRF-4, interferon regulatory factor 4; OCT, optimal cutting temperature compound; PHA, phytohemagglutinin; TPA, 12-O-tetradecanoylphorbol-13-acetate

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In this study, we examine IL-9 expression and the frequency of PU.1-positive T lymphocytes (Th9 lymphocytes) infiltrating into positive patch-test reactions to a variety of allergens. We identify allergen-specific IL-9 production by PBMCs (peripheral blood mononuclear cells) from a group of seven patients with positive patch tests to nickel. Our data show that IL-9 and related cytokine gene expression as well as PU.1⁺CD4⁺ T cells are significantly increased in positive patch-test biopsies from these allergic patients. *In vitro* experiments show IL-9 synergizing with signature cytokines from Th2 lymphocytes and regulating Th1 lymphocyte allergen-specific responses. The role of IL-9 found in our *in vitro* human data was confirmed using an *in vivo* mouse model, by studying contact hypersensitivity (CHS) to DNFB in IL-9 gene-targeted mice (IL-9^{-/-}). This mouse model confirmed the regulatory role of IL-9 in CHS.

RESULTS

The genes encoding IL-9 and Th9-associated transcription factor, PU.1, are elevated in ACD

We measured IL-9 and other cytokine genes associated with ACD in positive patch tests, as well as in paired normal skin.

The mean relative gene expression of IL-9 (Figure 1a) was elevated on average fivefold (range 2–18) higher than in paired normal skin. This was equivalent to the mean change in gene expression for IFN- γ , IL-4, and IL-17A. We also studied Th9-associated transcription factors PU.1, ETS-1, IFN regulatory factor 4, and GcN5 (Oikawa and Yamada, 2003; Refaat *et al.*, 2011; Goswami and Kaplan, 2012), which were all similarly elevated two- to threefold (range 1.5–8) higher than in paired control skin (Figure 1b).

Th9 lymphocytes, but not CD8⁺/PU.1⁺ lymphocytes, are detectable in the inflammatory infiltrate of ACD

Immunohistochemistry was performed for Th9 transcription factor PU.1 and T-cell surface markers (CD3, CD4, or CD8) in positive patch-test biopsies from five patients with ACD to different allergens. Either CD3⁺/PU.1⁺ or CD4⁺/PU.1⁺ double-positive cells were readily identifiable in the cellular infiltrates of ACD, present in both the dermis and the epidermis (Figure 1c). Of the total population of infiltrating CD3⁺/CD4⁺ T cells, ~4% were PU.1⁺ (Figure 1d and e). There were no CD8⁺/PU.1⁺ double cells detected in ACD

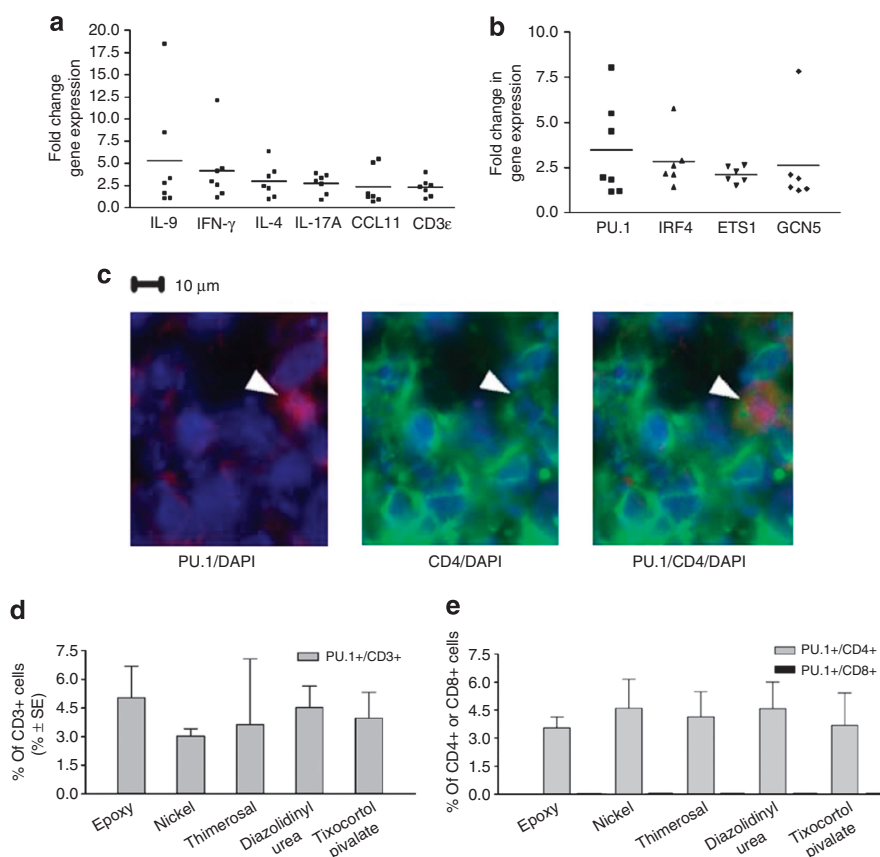


Figure 1. Increased expression of IL-9 and associated genes in allergic contact dermatitis (ACD) and the detection of Th9 cells in allergic contact dermatitis.

(a) Mean relative gene expression of IL-9 was elevated on average fivefold (range 2–18) higher than that in paired control skin. IL-9 increase is similar to increases in IFN- γ , IL-4, IL-17A, CCL11, and CD3 ϵ . (b) Mean relative gene expression of IL-9/Th9-associated transcription factors PU.1, IRF4, ETS-1, and GcN5 was two- to threefold (range 1.5–8) greater than that in paired control skin. Sections of skin biopsy specimens from a representative sample. (c) Stained slides from an ACD patient were stained with anti-PU.1 (red) and anti-CD4 (green); nuclei are counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Stained T lymphocytes were identified as (d) PU.1⁺/CD3⁺ or (e) PU.1⁺/CD4⁺. No PU.1⁺/CD8⁺ cells were identified. To quantify cell populations, 12 fields from each double-stained section were counted with a mean of 40 infiltrating cells per field. Mean \pm SEM is depicted. For gene expression studies, skin biopsy samples were taken from positive patch tests to nickel or cobalt from seven different patients (ACD6–12 in Table 1). For the immunohistochemistry, skin biopsy specimens were taken from five different positive patch tests in five different patients (ACD1–5 in Table 1).

(Figure 1e). No PU.1-positive T cells were detectable in the normal skin samples that were examined (data not shown).

Allergen-specific IL-9 production *in vitro* by PBMCs from nickel allergic patients

Next, we investigated nickel-specific cytokine production in lymphocytes from seven different nickel-allergic patients. We measured levels of IL-9, IFN- γ , IL-2, and IL-4 in culture supernatant in response to *in vitro* challenge with nickel or cobalt chloride, a nonallergic metal salt as confirmed by patch test. In several patients, we observed a robust dose-dependent production of IL-9 in response to increasing doses of nickel (Figure 2a). In contrast, PBMCs derived from two patients that were tolerant of nickel (negative patch tests), their PBMCs did not produce detectable IL-9 in response to nickel challenge *in vitro* (Figure 2a). To confirm that PBMCs have a similar capacity for the production of IL-9 in the nickel-allergic and -tolerant patients, we studied mitogen-induced IL-9 production (Figure 2b). Both groups produced high levels of IL-9 in response to phytohemagglutinin stimulation, which was equivalent between the two groups. In the nickel-allergic patients who had negative patch tests to cobalt chloride there was no IL-9, or other cytokines, produced in response to the irrelevant control metal salt, cobalt chloride (data not shown). From the dose-response curves of nickel-specific cytokine production, we plotted the peak cytokine production from each of the seven nickel-allergic patients (Figure 2c). The mean peak cytokine production of both IFN- γ and IL-9 was significantly higher than IL-4. These data indicate that allergen-specific IL-9 production is at a similar level as IFN- γ in patients with ACD to nickel.

Characterization of antigen-presenting cell pathways for recognition of nickel-specific IL-9 production

In order to elucidate how Th9-specific antigens are being processed and presented, blocking studies were performed. A mAb specific for HLA-DR, associated with the major histocompatibility complex class II (MHC II) pathway, significantly attenuated nickel-specific IL-9 production, whereas anti-HLA-A, -B, or -C had no significant effect on IL-9 (Figure 3). Similarly, pre-incubation of PBMCs with chloroquine, before nickel stimulation, also significantly attenuated nickel-specific IL-9 (Figure 3). These data indicate that allergen-induced IL-9 production is dependent on the MHC class II pathway and requires processing. This is consistent with the *in situ* data in which we observed CD4 + PU.1 + T lymphocytes, but not CD8 + PU.1 + T lymphocytes in ACD (Figure 1).

Regulatory role of IL-9 in ACD

We next investigated whether IL-9 modulates nickel-specific IFN- γ production. As expected, exogenous IL-4 significantly decreased nickel-specific IFN- γ production by PBMCs from a nickel-allergic patient, and exogenous IL-9 did not attenuate nickel-specific IFN- γ production (Figure 4a). When exogenous IL-9 and IL-4 were added simultaneously to lymphocyte cultures, the inhibitory effects of IL-4 on IFN- γ production were dominant and were not influenced by the presence of

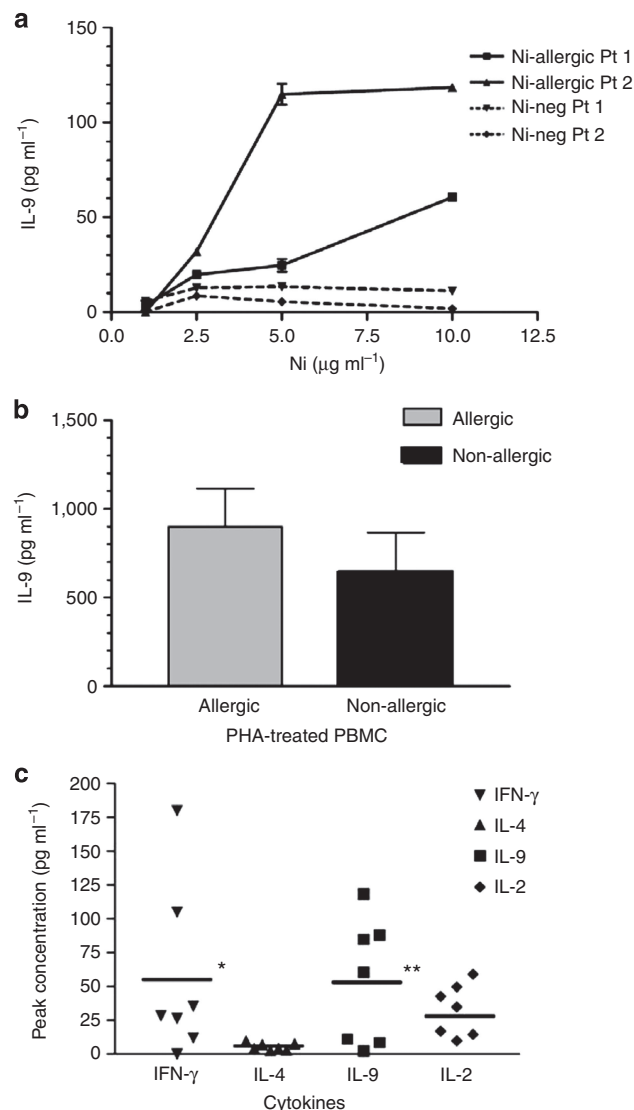
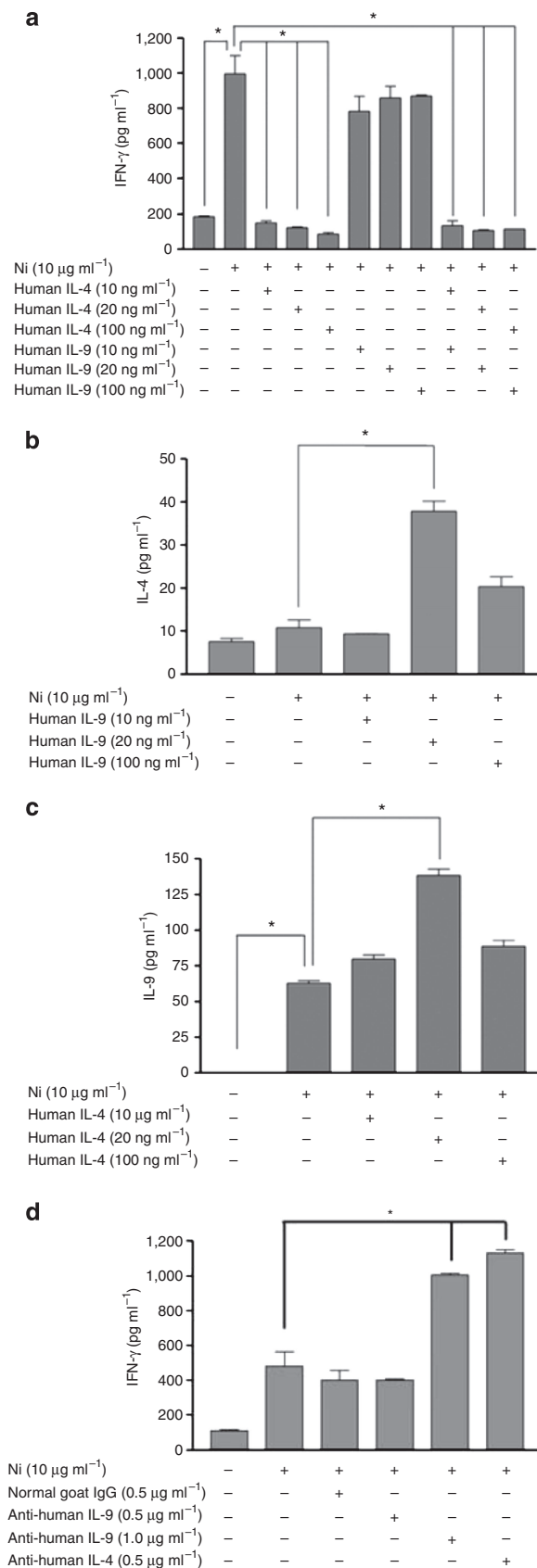


Figure 2. Allergen-specific IL-9 production by peripheral blood mononuclear cells (PBMCs) from nickel-allergic patients. PBMCs were isolated from seven different patients with allergic contact dermatitis to nickel (Table 1) and cultured with nickel chloride ($1\text{--}10\text{ }\mu\text{g ml}^{-1}$ of culture medium) for 96 hours. Supernatants were harvested, and the following cytokines were assayed in cell-free culture supernatants: IL-9, IFN- γ , IL-4, and IL-2 (data depicted represent mean \pm SD of samples assayed in triplicate). (a) Dose-response curve to nickel titration from two nickel-allergic patients and two nickel-tolerant patients. (b) IL-9 production from the same two nickel-allergic and -tolerant patients, demonstrating that both groups can produce IL-9 in an equivalent manner after phytohemagglutinin (PHA) stimulation. (c) Scatter plot represents the peak cytokine production from the seven allergic patients. The scatter plot of peak cytokine production (horizontal bar represents mean value) for both IFN- γ and IL-9 was significantly higher than that of IL-4 ($P < 0.001$), with the average peak IL-9 production similar to IFN- γ ($P > 0.05$).

IL-9. We also studied the cross-regulation of IL-9 and IL-4 during allergen-specific responses (Figure 4b and c). Exogenous IL-9 augmented nickel-specific IL-4 (Figure 4b) and exogenous IL-4 augmented nickel-specific IL-9 (Figure 4c). These data suggest that there is synergy between Th2 and Th9



lymphocytes via their cytokines, and also suggest an indirect regulatory role for IL-9 in ACD. Whereas IL-9 itself does not attenuate IFN- γ production by Th1 lymphocytes, this cytokine augments the production of IL-4, which is a known inhibitory cytokine for Th1 lymphocytes (Mosmann *et al.*, 1986; Cher and Mosmann, 1987; Yip *et al.*, 1999). The addition of anti-sera to block either IL-9 or IL-4 in cultures of nickel-stimulated PBMCs augmented IFN- γ production, again indicating a regulatory function for IL-9 (Figure 4d).

The regulatory role of IL-9 in an *in vivo* model of contact hypersensitivity

To further confirm our human *in vitro* data, we studied contact hypersensitivity in IL-9 gene-targeted mice (IL-9^{-/-}), and compared their ear swelling responses with those of WT mice

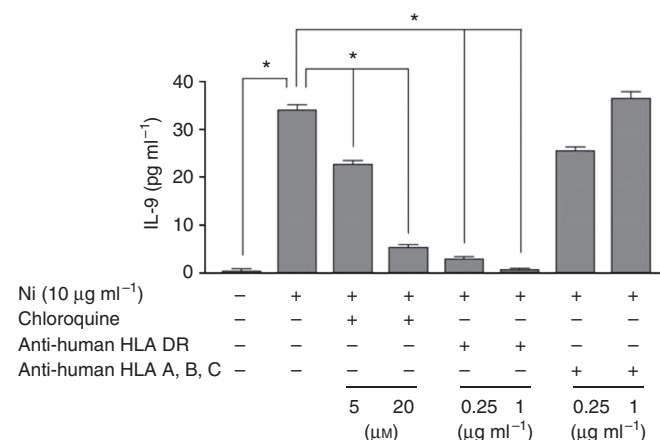


Figure 3. IL-9 production depends on exogenous antigen presentation to Th9 lymphocytes. IL-9 production was measured 96 hours after stimulation of peripheral blood mononuclear cells (PBMCs) with medium containing nickel chloride (10 μg ml⁻¹). Samples are incubated with either media, anti HLA DR mAb (0.25 μg ml⁻¹ and 1 μg ml⁻¹), anti-HLA-A, B, C mAbs (0.25 μg ml⁻¹ and 1 μg ml⁻¹), or chloroquine (5 μM and 20 μM) 2 hours prior to stimulation with nickel. Antibodies to HLA-A, B, C had little effect on IL-9 production, whereas Abs to HLA-DR or incubation with chloroquine resulted in significantly reduced IL-9 production. Mean \pm SD of samples assayed in triplicate is depicted. Our results suggest that the processing of nickel via the exogenous pathway of antigen presentation is necessary for PBMC production of IL-9. The experiment was repeated with PBMCs from three different patients, and found to be reproducible.

Figure 4. IL-4, but not IL-9, directly regulates IFN- γ during allergen-specific Th1 activation *in vitro*. (a) Peripheral blood mononuclear cells (PBMCs) from a nickel-allergic patient were cultured in medium alone or in medium containing nickel chloride; the indicated doses of IL-9 alone, IL-4 alone, or IL-9 and IL-4 together were added to the nickel chloride-stimulated PBMCs. Supernatants were collected 96 hours after nickel stimulation. (b) IL-4 secretion was also measured after addition of IL-9. (c) IL-9 was measured in those culture conditions, which had added IL-4. (d) Specific blocking anti-sera were added to the nickel culture, and IFN- γ was assayed using multiplex ELISA. Data depicted represent mean \pm SD of samples assayed in triplicate. This experiment was repeated with PBMCs three times and was found to be reproducible. The data in (a-d) represent PBMCs from distinct nickel-allergic patients.

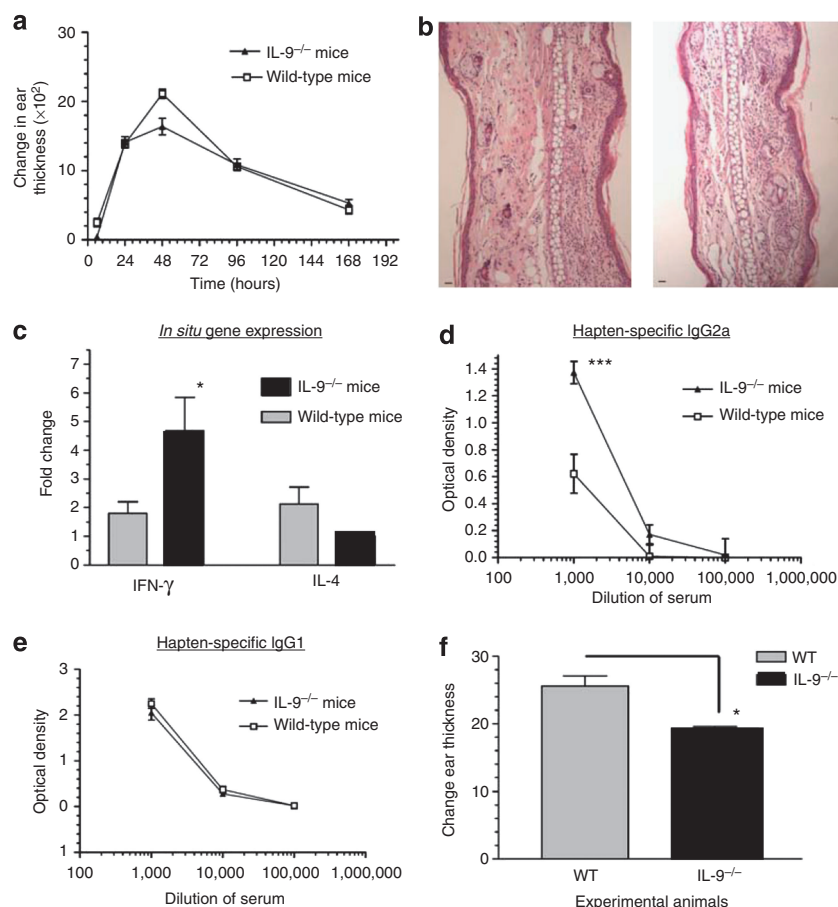


Figure 5. The regulatory role of IL-9 in mouse contact hypersensitivity. (a) Ear swelling response of wild type (WT) or IL-9^{-/-} mice ($n = 5$ group⁻¹) was measured over time. (b) Ear punches taken 24 hours after elicitation are stained with hematoxylin and eosin stain; shown are ears from WT (left) and IL-9^{-/-} mice (right). (c) Gene expression was measured by quantitative PCR from ear skin 24 hours after elicitation. Mean \pm SD fold change in gene expression is shown ($n = 4$ mice/group, $*P < 0.05$). (d) Sera ($n = 5$ mice/group) from WT or IL-9^{-/-} mice 21 days after sensitization were measured for DNP-specific IgG1 or (e) IgG2a by ELISA. Levels of DNP-specific Ab from pre-bleed sera (prior to sensitization) were subtracted to calculate the net DNP-specific response. Data shown represent mean \pm SD ($***P < 0.001$). (f) Irritant contact dermatitis to croton oil in WT and IL-9^{-/-} mice; data indicate mean \pm SD ($P < 0.0023$).

of the same H-2^d background. When compared with WT mice, IL-9^{-/-} mice exhibited slightly diminished ear swelling responses at 48 hours (Figure 5a). Histological analysis of ear sections taken 24 hours after hapten challenge from either WT or IL-9^{-/-} mice exhibited qualitatively similar pathologic findings (Figure 5b). However, analysis of *in situ* cytokine gene expression revealed that during CHS reaction IL-9^{-/-} mice showed enhanced Th1 and diminished Th2 gene expression, whereas WT have a balanced Th1–Th2 profile (Figure 5c). The mean fold change in IFN-γ gene expression was significantly greater in IL-9^{-/-} mice compared with the WT mice ($P < 0.05$, analysis of variance). To better understand the systemic effects of the role of IL-9 in contact hypersensitivity, we studied dinitrophenyl-specific IgG1 and IgG2a in the sera of sensitized mice 3 weeks after sensitization with topical DNFB. Dinitrophenyl-specific IgG1 (Th2 driven) was similar in WT and IL-9^{-/-} mice, and there was significant enhancement of IgG2a (Th1 driven) in IL-9^{-/-} mice compared with wild-type mice (Figure 5d and e). These data indicate that IL-9 exhibits a regulatory role in ACD and also that compensatory

inflammatory mechanisms operate in the absence of IL-9, resulting in a relatively preserved ear swelling response in which Th1 lymphocytes have a more important role both locally and systemically. To further study the response to inflammatory agents, we challenged naive WT and IL-9^{-/-} mice with the topically applied irritant, 12-O-tetradecanoyl-phorbol-13-acetate. After 24 hours of application, IL-9^{-/-} mice exhibited significantly less ear swelling compared with WT control mice ($t = 4.069$, unpaired t -test, $P < 0.0023$).

DISCUSSION

ACD is a result of a complex network of antigen-presenting cell–T-cell interactions, and represents a balance between effector subsets and regulatory subsets (Gober and Gaspari, 2008). Whereas CD8⁺ T lymphocytes are effector cells that mediate tissue damage during the elicitation phase of ACD, the CD4⁺ T-lymphocyte subset has a regulatory role in ACD of secreting an immune suppressive milieu of cytokines (Gocinski and Tigelaar, 1990; Bour *et al.*, 1995; Martin *et al.*, 2000; Vocanson *et al.*, 2006). In human positive

Table 1. Allergen and patient data

Patient no. ¹	Age (years)/gender/race ²	Allergen/scoring/duration (h) ³	Studies ⁴
ACD1	59/M/W	Epoxy resin/+/48	ICC
ACD2	68/M/W	Thimerosal/+/+/96	ICC
ACD3	60/M/A	Diazolidinyl urea/+/+/48	ICC
ACD4	46/F/AA	Tixocortolpivalate/+/+/96	ICC
ACD5	72/F/W	Nickel sulfate/+/72	ICC
ACD6	54/F/W	Nickel sulfate/+/+/72	PCR
ACD7	38/F/W	Nickel sulfate/+/+/72	PCR
ACD8	29/F/W	Nickel sulfate/+/+/72	PCR
ACD9	41/F/W	Nickel sulfate/+/72	PCR
ACD10	54/F/W	Nickel sulfate/+/+/72	PCR
ACD11	43/M/W	Cobalt chloride/+/72	PCR
ACD12	64/F/W	Nickel sulfate/+/+/72	PCR
ACD13	39/F/A	Nickel sulfate/+/48	ELISA
ACD14	62/F/AA	Nickel sulfate/+/+/48, Cobalt chloride/+/+/48	ELISA
ACD15	58/F/W	Nickel sulfate/+/+/72, Neomycin/+/72, Thimerosal/+/72	ELISA
ACD16	29/F/W	Nickel sulfate/+/+/48	ELISA
ACD17	58/F/W	Nickel sulfate/+/+/72, Cobalt chloride/+/+/72	ELISA
ACD18	25/F/W	Nickel sulfate/+/+/72, Cobalt chloride/+/+/72	ELISA
ACD19	58/F/AA	Nickel sulfate/+/72, Cobalt chloride/+/+/72	ELISA

¹Patient no. refers to allergic contact dermatitis patients no. ACD1–19.

²Race: A, Asian; AA, African American; W, white (Caucasian).

³Patch-test scoring system: 0, negative; +, erythema; some induration (low-level reaction); ++, erythema spreading and more marked induration (moderate reaction); +++, erythema, marked induration, and blisters (extreme reaction).

⁴Studies refers to PCR (real-time PCR), ICC (immunocytochemistry), ELISA (studies on culture supernatants from allergen-stimulated lymphocytes).

patch-test reactions, a multitude of cytokines can be identified (Zhao *et al.*, 2009), suggesting that a variety of Th subsets may be involved in ACD. Our data investigating human skin biopsies from ACD to nickel and other clinically relevant allergens (Table 1) are consistent with this. We found that genes encoding several cytokines were increased in human ACD (Figure 1). However, we also identified increased levels of IL-9 and the Th9-associated transcription factors, PU.1, ETS-1, IFN regulatory factor 4, and GcN5 at similar levels as these well-known ACD-associated cytokines. These results suggest that Th9-derived IL-9, as well as other classically associated cytokines, have a role in human ACD.

We demonstrate by using biopsies from positive patch tests that allergen-specific CD4⁺/PU.1⁺ T cells, but not CD8⁺/PU.1⁺ T cells, are detectable in the efferent phase of ACD

(Figure 1). This is consistent with a regulatory, rather than a pathogenic, role for IL-9-producing T cells in ACD. It has been shown that lymphocytes from patients with positive patch tests (i.e., ACD) to metal salts produce both Th1-associated IFN- γ as well as Th2-associated IL-4 and IL-13 upon culture with the appropriate allergen (Minang *et al.*, 2006). When measuring allergen-specific cytokine production of *ex vivo* cultured PBMCs from ACD patients, we find that the allergen-specific IL-9 production by *ex vivo* PBMCs is augmented in allergic patients, but not in nonallergic control patients (Figures 2–4). We were able to detect nickel-specific IL-4 at low levels, whereas IFN- γ and IL-9 showed a robust response to nickel (Figure 2). We were not able to identify CD4⁺/PU.1⁺ T cells in normal human skin (data not shown). However, this cell type constituted ~4% of the inflammatory infiltrate of ACD and we noted elevated IL-9 transcripts in ACD compared with paired normal skin (Figure 1). These data suggest that the IL-9-producing CD4⁺/PU.1⁺ T cells migrate into the skin of ACD.

Xenobiotic chemicals are thought to be chemically reactive with host proteins, by forming haptens, which are presented by migratory cutaneous antigen-presenting cells in the local lymph node (Kaplan *et al.*, 2012). Haptens can be recognized by class I MHC-restricted CD8⁺ T lymphocytes, or class II MHC-restricted CD4⁺ T lymphocytes. Studies of processing/presentation of nickel to reactive T lymphocytes suggested two pathways for nickel recognition: one involves a processing independent pathway in which nickel binds to peptides already loaded into MHC molecules, and the other pathway involves a processing-dependent pathway in which nickel binds to membrane or exogenous proteins, which are then taken up by an antigen-presenting cell for processing and presentation to reactive T lymphocytes (Budinger and Hertl, 2000). We find that production of nickel-specific IL-9 is blocked by anti-class II MHC mAbs but not anti-class I MHC antibodies supporting the notion that Th9 lymphocytes, but not CD8⁺/PU.1⁺ lymphocytes, are the predominant source of IL-9 (Figure 3). We also find that chloroquine, an agent that interferes with the acidification of the endosome and hence antigen processing (Lombard-Platlet *et al.*, 1993), interferes with nickel-specific IL-9 production, which supports the processing-dependent pathway of nickel presentation.

In some model systems, neutralization of IL-9 enhances IFN- γ production in PBMCs from patients with latent tuberculosis infections, suggesting that this cytokine blocks Th1 cytokine secretion (Wu *et al.*, 2008). IL-9 can also enhance IFN- γ production and suppress Th2 responses during active tuberculosis infections (Finiasz *et al.*, 2007). In our *in vitro* assays, exogenous IL-9 did not inhibit nickel-specific IFN- γ production (Figure 4); however, blockade of endogenous IL-9 by using a specific anti-serum enhanced IFN- γ production. Our data indicate that IL-9 acts to regulate allergen-specific Th1-lymphocyte responses directly (Figure 4d) and indirectly by enhancing IL-4 production (Figure 4b) by Th2 lymphocytes. IL-9 gene expression has been implicated in ACD to the hair dye paraphenylenediamine. PBMCs from allergic, but not tolerant individuals, increased IL-9 gene expression upon

exposure to this chemical *in vitro*, suggesting that this cytokine has a role in ACD (Coulter *et al.*, 2010).

Further experiments in this study demonstrate a regulatory role of IL-9 in a mouse model of CHS to the strong Th1-polarizing hapten DNFB. In mouse models of ACD, using strong lipophilic haptens, Th1 lymphocytes positively regulate ACD, and have a role in promoting an inflammatory response via IFN- γ production and other pro-inflammatory cytokines (Saulnier *et al.*, 1995; Xu *et al.*, 1996). In cytokine gene-targeted mice, IFN- γ as well as IL-4 are necessary for sensitization and elicitation, suggesting that both Th1 and Th2 lymphocytes have a role in murine ACD (Saulnier *et al.*, 1995; Xu *et al.*, 1996; Campos *et al.*, 2003). Previous studies of protein contact dermatitis in a mouse model demonstrated that Th9 lymphocytes are detectable in the afferent phase of sensitization to topical polypeptides in BALB/c mice, a phenomenon associated with Th2-dominant immune response (Lin *et al.*, 2012). Whereas past work has focused on the effector functions of IL-9 in allergic inflammation and the role of IL-9 in contact dermatitis to polypeptides, our study of CHS in IL-9^{-/-} mice addresses its role in the sensitization/elicitation phases of this allergic reaction.

Our data indicate that CHS to the Th1-polarizing hapten DNFB can occur in the absence of IL-9, but its inflammatory characteristics are altered. We find that there is an enhanced Th1 response locally, as well as enhanced hapten-specific IgG2a found in the sera of IL-9^{-/-} mice (Figure 5d and e). It has been established that IgG2a (enhanced in IL-9^{-/-} mice) is dependent on Th1-derived cytokines (Jurado *et al.*, 1989), whereas IgG1 (not enhanced in IL-9^{-/-} mice) is dependent on Th2-derived cytokines such as IL-4 (Coffman *et al.*, 1989). Our histologic analysis of skin challenge sites of DNFB-sensitized mice did not reveal quantitatively significant differences in the quality of the inflammatory infiltrate in wild-type or IL-9^{-/-} mice (Figure 5b). We noted T lymphocytes, neutrophils, and mast cells in both groups of experimental animals. For the hapten DNFB, it is likely that the loss of the pro-inflammatory effects of IL-9 may have been mitigated by enhanced Th1 lymphocyte-derived help in the form of IFN- γ , and possibly other cytokines, hence a relatively preserved ear swelling response in the IL-9^{-/-} mice (Figure 5a). In contrast, under induction of irritant contact dermatitis by 12-O-tetradecanoylphorbol-13-acetate, ear swelling inflammatory responses were significantly decreased in IL-9^{-/-} mice compared with WT mice. We hypothesize that this occurs because (in the absence of inflammatory effects of IL-9 in knockout mice) the T-cell compensatory mechanisms observed in ACD (Figure 5a) are not present in irritant contact dermatitis (Figure 5f).

In total, these *in vivo* data are consistent with our *in vitro* data in humans, showing that Th9 lymphocytes modulate allergen-specific IFN- γ production by Th1 lymphocytes. Our study contributes to the emerging evidence that Th9 has an important role in allergic skin reactions, and provides evidence that Th9 and its associated cytokine, IL-9, represent a potential target for immunotherapy in the treatment of non-atopic allergic skin diseases.

MATERIALS AND METHODS

Patients

Patients being evaluated for ACD were recruited from University of Rome and the University of Maryland, Baltimore (Table 1). Written informed consent was obtained from each patient. The study was approved by the local institutional review committee and followed the Declaration of Helsinki Principles for research involving human subjects. The standard North American Series (Dormer Laboratories, Toronto, ON, Canada) were applied in 8 mm Finn Chambers (Epitest Oy, Tuusula, Finland) to upper back of each patient using standard methods. Results were interpreted with the standard grading system (0 to 3+) at 48 and 72 hours indicated in Table 1. Paired biopsy samples of positive patch-test sites and normal control skin were collected and snap-frozen then stored at QUOTE 80°C before RNA extraction. Patch test-positive biopsies were obtained from additional patients and frozen in OCT compound for immunohistochemistry studies. Tonsil sections are used as negative and positive controls for immunohistochemistry.

Real-time PCR

RNA extraction, complementary DNA synthesis, and quantitative PCR were performed as previously described (Zhao *et al.*, 2009). Primer reference sequence accession number and positions (SABiosciences, Valencia, CA) are as follows: IL-9: NM_000590.1 (Reference position: 353); CD3 ϵ : NM_007648.4 (Reference position: 480); PU.1: NM_003120.2 (Reference position: 805); ETS-1: NM_005238.2 (Reference position 1561); IFN regulatory factor 4: NM_002460.3 (Reference position 1303); GcN5 (KAT2A): NM_021078.2 (Reference position 1664); eotaxin gene (CCL11): NM_002986.2 (Reference position: 285); IL-4: NM_000589.2 (Reference position:122); IFN- γ : NM_000619.2 (617); IL-17A: NM_010552.3 (Reference position:111). For mouse cytokine genes: IL-4: NM_021283 (Reference position: 46); IFN- γ : NM_008337 (Reference position: 309). Levels of 18S mRNA are used to normalize cytokine gene expression levels before calculating fold changes among patch test-positive and normal skin. For mouse tissue, the CHS skin was normalized to normal mouse ear skin.

Immunohistochemistry

ACD skin biopsies were embedded in OCT, immediately frozen in liquid nitrogen, followed by sectioning and fixation in acetone. Slides were incubated with rabbit anti-human PU.1 polyclonal antibody (Cell Signaling Technology, Danvers, MA), followed by AlexaFluor594 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). For double labeling of T cells and T-cell subsets, the slides were then incubated with fluorescein isothiocyanate-labeled monoclonal mouse anti-human CD3, CD4, or CD8 antibodies (BD Pharmingen, San Diego, CA). The slides were examined by a fluorescence microscope and two color digital images were recorded. On average, 12 fields from each double-stained section were counted with a mean of 40 infiltrating cells per field.

In vitro cytokine secretion and hapten-specific antibody ELISA

PBMCs were isolated from allergic patients and nonallergic control subjects using Accuprep (Accurate Chemical & Scientific, Westbury, NY). PBMCs were cultured at a concentration of $2 \times 10^5 \text{ ml}^{-1}$ in AIM-V medium with 10% fetal bovine serum in triplicate. Cells were cultured with the pan T-cell mitogen phytohemagglutinin $20 \mu\text{g ml}^{-1}$, nickel (1.25, 2.50, 5.00, or 10.00 ml^{-1}), cobalt (1.25, 2.50, 5.00, or

10.00 $\mu\text{g ml}^{-1}$), or culture medium alone, and were then incubated at 37°C for 96 hours. Next, supernatants were collected and *in vitro* cytokine secretion for IFN- γ , IL-2, and IL-4 was measured by Luminex assay with MILLIPLEX MAP Kit (Millipore, Billerica, MA) and were assessed for IL-9 production with LEGEND MAX human IL-9 ELISA kit (Biolegend, San Diego, CA) (limit of detection for IL-9 is 1 pg ml^{-1}) according to the manufacturer's instructions (Pickering *et al.*, 2002). ELISA to detect hapten-specific antibody responses was performed as previously described (Thatcher *et al.*, 2006).

Blocking IL-9 production *in vitro*

PBMC were pretreated with either anti-HLA DR (Mouse IgG2 α , κ , mAb, L243, Biolegend) or HLA-A,B,C (Mouse IgG2 α , κ , mAb, W6/32, Biolegend) at a concentration of 0.25 $\mu\text{g ml}^{-1}$ or 1 $\mu\text{g ml}^{-1}$. In parallel, they were pre-incubated with chloroquine (Sigma, St. Louis, MO) at a concentration of 5 μM or 20 μM for 2 hours, washed extensively, then incubated with medium containing nickel chloride or medium alone at 37°C for 96 hours. Supernatants were collected for *in vitro* cytokine IL-9 detection.

Regulation of IFN- γ production *in vitro* by IL-9 or IL-4

PBMCs were incubated with recombinant human IL-9 (R&D Systems, Minneapolis, MN); human IL-4 (*Escherichia coli* derived, R&D Systems) at a concentration of 10, 20, or 100 ng ml^{-1} ; or combinations of IL-4 and IL-9 during the stimulation with metal nickel chloride (10 $\mu\text{g ml}^{-1}$) or medium, and then incubated at 37°C for 96 hours. Supernatants were collected for *in vitro* cytokine analysis of IFN- γ , IL-4, and IL-9.

Blocking IL-4 or IL-9 *in vitro*

PBMCs were incubated with anti-human IL-9 (polyclonal goat IgG, R&D Systems) at a concentration of 0.5 $\mu\text{g ml}^{-1}$ or 1.0 $\mu\text{g ml}^{-1}$; anti-human IL-4 (polyclonal goat IgG, R&D systems) at a concentration of 0.5 $\mu\text{g ml}^{-1}$; or normal goat IgG (polyclonal, R&D Systems) at a concentration of 0.5 $\mu\text{g ml}^{-1}$ during the stimulation with metal nickel chloride (10 $\mu\text{g ml}^{-1}$) or medium and then incubated at 37°C for 96 hours. Supernatants were collected for *in vitro* cytokine analysis of IFN- γ .

Contact hypersensitivity assay

DNFB CHS experiments with WT and IL-9 $^{-/-}$ mice were conducted as previously described (Thatcher *et al.*, 2006). At challenge, both sides of the ears were treated with DNFB. Ear swelling was calculated by subtracting the baseline ear thickness from ear thickness 24 hours after DNFB challenge (Minang *et al.*, 2006). The CHS assay was repeated twice, and found to be reproducible. For irritant contact dermatitis, 1% croton oil in acetone was applied to mouse ears as previously described (Schwarz *et al.*, 1993), and ear swelling was measured at 24 hours. Skin biopsy specimens were taken from the pinna of the ear in selected animals at 24 hours after hapten challenge. These biopsies were paraffin embedded, sectioned onto glass slides, stained with hematoxylin-eosin, and examined and photographed using a E600 Nikon microscope equipped with a Spot Digital camera (Diagnostic Instruments, Sterling Heights, MI).

Experimental mice

The experimental mice were either WT BALB/c mice (H-2 d) (Jackson Labs, Bar Harbor, ME) or IL-9 knockout mice (IL-9 $^{-/-}$) generated on

the BALB/c (H-2 d) background in the laboratory of Dr Flavell. The experimental animals were kept in standard housing conditions, were fed mouse chow *ad libitum*, and were exposed to a 12 hour light/dark cycle. All experimental procedures were reviewed and approved by the Yale University Institutional Animal Care and Use Committee.

Statistical analysis

The data were expressed as mean \pm SD of indicated numbers of experiments with GraphPad Prism 3.03 (GraphPad Software, La Jolla, CA). The Pearson's *r* correlation analysis test was performed with GraphPad InStat 3.06 (GraphPad Software). The unpaired *t*-test is performed for cytokine release; *P*-values <0.05 were considered significant. Analysis of variance was used to compare the WT with IL-9 $^{-/-}$ mice for dinitrophenyl-specific Ig production.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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